GENETICALLY ENGINEERED RABIES RECOMBINANT VACCINE FOR IMMUNIZATION OF STRAY DOGS AND WILDLIFE

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CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority in part under 35 U.S.C. §119 based upon U.S. Provisional Patent Application No. 60/191,510 filed March 23, 2000.

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GOVERNMENT RIGHTS IN THE INVENTION

This invention was made in part with government support under Grant numbers AI45097, 5RO1A145097-02, and AI41544 awarded by the National Institutes of Health. The government has certain rights to the invention.

FIELD OF INVENTION

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The present invention relates to the field of biotechnology and immunology, and more particularly to the design of recombinant rabies virus vaccines by replacing the glycoprotein of a non-neuroinvasive rabies strain with that of a street rabies virus and/or by constructing a recombinant rabies virus expressing a pro-apoptotic protein, thereby eliciting an optimal immunoprotective response against rabies virus.

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BACKGROUND OF THE INVENTION

The rabies virus is a rhabdovirus, a nonsegmented RNA virus with negative sense polarity. The genome codes for five proteins: 3 internal proteins are an RNA-dependent RNA polymerase (L), a nucleoprotein (N) and a phosphorylated protein

(NS); a matrix protein (M) located on the inner side of the viral envelope and an

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external surface glycoprotein (G). (Dietzschold, B. & Ertl, H. Cricial Rev. in Immunology 10:427-439, 1991). The virus is transmitted through broken skin by the bite or scratch of an infected animal. This exposure to rabies virus results in its penetration of peripheral unmyelineated nerve endings, followed by spreading through retrograde axonal transport, replication occurring exclusively in the neurons, and finally arrival in the central nervous system (CNS). Infection of the CNS causes cellular dysfunction and ultimately death. (Rupprecht, C.E., & Dietzschold, B. Lab Invest. 57:603, 1987). Since rabies virus spreads directly from cell to cell, it evades immune recognition. (Clark, H.F. & Prabhakar, B.S., Rabies, In: Olson R.G., et al., eds., Comparative Pathology of Viral Disease, 2:165, Boca Raton, FL: CRC Press, 1985). Therefore, in order to effectively prevent disease, immunization should inhibit the ability of the virus to enter the cells.

Rabies is a worldwide public health problem. There is no successful treatment of clinical rabies, the outcome is almost always fatal. The rabies virus is maintained in many animal reservoirs, wildlife as well as domestic. Therefore, in order to eliminate pathogenesis in humans, as well as livestock, it is necessary to eliminate The most efficient vaccination protocol would be the these viral reservoirs. development of oral vaccines that induce a long-lasting protection against subsequent exposure to the rabies virus. It has been shown that certain rabies virus variants, such as SAG-2 and SAD B19, or a vaccinia rabies virus glycoprotein recombinant virus are effective vaccines that can be used for the oral vaccination of certain wildlife, such as foxes and raccoons. (Rupprecht, C.E., et al., Emerg. Infect. Dis., 1:107-114, 1995). However, these vaccines do not induce sufficient protective immunity when administered orally to dogs, and it is the domestic dog that is the principal host and major vector of rabies throughout the world. (Fekadu, M., Canine Rabies, In: Baer, G.M., ed. The Natural History of Rabies, 367-378, Boca Raton, FL: CRC Press, 1991; Wang, Y. & Walker, P.J., Virology 195:719-731, 1993).

In developing countries, dogs are responsible for ~94% of human rabies deaths. For example, in Thailand, which has an estimated population of 7 million dogs, one of every 961 dogs was found to test positive for rabies. Assuming a mean vaccination cost of one U.S. dollar per dog, the minimum spending for dog vaccination in developing countries would be around U.S. \$50,000,000. (Meslin,



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F.X., et al., In: *Lyssaviruses*, Rupprecht, C.E., et al., eds., Springer-Verlang, Berlin, Heidelberg, New York, 1-26, 1994).

In the Americas, the rabies situation is much more complex than that of developing countries. Reservoirs of rabies exist in many diverse wild animal species, in the United States these resevoirs accounted for nearly 93% of the 8513 reported cases of rabies in 1997. (Rupprecht, C.E., et al., *Emerging Infectious Diseases* 1(4): 107-114, 1995). The most frequently reported rabid wildlife species are raccoons (50.5%), followed by skunks (24.0%). (Rupprecht, C.E., et al., *Emerging Infectious Diseases* 1(4): 107-114, 1995). Outbreaks of rabies infections in these terrestrial mammals are found in broad geographic areas across the United States. For example, raccoon rabies affects an area of more than 1 million square kilometers from Florida to Maine.

Oral immunization of stray dogs and wildlife against rabies is the most effective method to control, and eventually eradicate, rabies. (Winkler, W.G. & Bogel., K. Sci. Amer., 266(6):86-92, 1992). In this regard, significant progress has been made in the development of oral rabies vaccines for the control of vulpine rabies. (Aubert, M.F.A., et al., Lyssaviruses, Rupprecht, C.E., et al., eds., Springer-Verlang, Berlin, Heidelberg, New York, 219-243, 1994). However, while oral immunization with conventional modified-live vaccines such as SAD B19, SAG-2, or poxvirus-rabies glycoprotein recombinant vaccines are very effective in foxes (Aubert, M.F.A., et al., Lyssaviruses, Rupprecht, C.E., et al., eds., Springer-Verlang, Berlin, Heidelberg, New York, 219-243, 1994), they do not immunize skunks or induce only low seroconversion by the oral route (Rupprecht, C.E., et al., J. Wildl. Dis. 99-102, 1990). Moreover, very high doses of these vaccines (> $10^{8.5}$ TCID₅₀) are necessary to induce protective immunity via oral immunization of dogs. (WHO Report of the 4th WHO consultation on oral immunization of dogs against rabies, Geneva, RabRes, 93:42, 1993). These findings make oral field vaccinations economically impractical. Therefore, in order to control wildlife rabies and rabies in stray dogs worldwide, more potent and cost effective oral rabies vaccines must be developed. There is a high demand for such vaccines. For example, based on previous experience that a minimal density of 20 vaccine-laden baits per square mile is sufficient for immunization of foxes (Aubert, M.F.A., et al., Lyssaviruses, Rupprecht, C.E., et al., eds., Springer-Verlang, Berlin, Heidelberg, New York, 2195

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243, 1994), more than 20 million doses of vaccine alone would be required for the control of the raccoon rabies enzootic in the Atlantic regions of the United States.

Vaccines prepared with antigenically conserved lab rabies virus strains may not be effective against those found in the wild, i.e., the street virus. There is a need for versatile vaccines suitable for both domestic animals and wildlife, which either serve as reservoirs for human rabies or are economically important species. Efforts have been made to protect free-ranging animals against virulent street virus challenge by oral consumption of a potent vaccine contained within an attractive bait. Yet concerns regarding residual virulence and ineffectiveness remain. Therefore, there exists a long felt need for a new generation of live rabies vaccines. The present invention describes a new generation of live rabies vaccines that has been developed using reverse genetics technology. (Schnell, M.J., et al., *EMBO* 13:4195-4203, 1994).

In addition to virus-neutralizing antibodies (VNA), which are believed to be the major immune effectors against rabies, rabies virus antigen-specific (CD4⁺) T helper cells and cytotoxic T cells (CD8⁺) (Cox, J.H., et al., *Infect. Immun.* 16:754-759, 1977), as well as innate mechanisms (Hooper, D.C., et al., *J. Virol.* 72:3711-3719, 1998), play an important role in the immune defense against rabies. The rabies virus glycoprotein (G) induces the production of VNA, while the cellular responses of CD4⁺ and CD8⁺ T cells are predominantly triggered by the internal rabies virus proteins; therefore, live rabies virus represents the best immunogen that will confer optimal protective immunity.

The extent of the immune response to immunization with a live virus vaccine is determined by the antigenic mass administered and produced after administration of the vaccine. Inoculation with a live, yet attenuated, virus will allow for the production of antigen in the absence of pathogenicity. The site of antigen production and presentation are also important factors that determine the potency of the vaccine. In this context, the fixed and street rabies virus variants differ substantially in their ability to replicate in neuronal versus non-neuronal cells (neuronal specificity index). (Morimoto, K., et al., *J. Neuro Virol.*, 6:373-381, 2000). The neuronal specificity index of any particular rabies virus variant is determined by its glycoprotein. The glycprotein is also the major viral protein that determines the host specificity of the strain. In this context, it is the rabies glycoprotein that carries the major determinants responsible for the pathogencity of the virus, as well as the determinants that trigger a

Serial No. 10/047,011

Lorin Ullmann

Page 6 of 28

Claim 13 (currently amended):

A system for inserting stack signature marking code segments into application software modules prior to compilation, said system cooperating with a compiler and comprising:

a control means operable by a user to indicate whether or not to insert stack signature marking code segments into application software modules; [[and]]

a code insertion means which, responsive to the operation of the control means, searches for entry points and exits points in application software modules and inserts stack signature marking code segments following each entry point and prior to each exit point into said application software modules[[.]];

a compiler means for producing one or more executable programs containing one or more executable re-entrant or object oriented programming code modules containing said inserted stack signing software; and

a debugger means configured to, upon execution of said executable re-entrant or object-oriented code modules, assign unique module identifier values to said code modules by said stack signing software, said stack signing software preventing module identifiers from having a same value for multiple instances of any re-entered or multiply instantiated code module, and to push onto said processing stack said stack signatures.

Claim 14 (currently amended):

The system of Claim [[14]] 13 wherein said control means comprises a global control means for indicating insertion of stack signature marking code segments are to be inserted into all application software modules to be compiled.

Claim 15 (currently amended):

The system of Claim [[15]] 13 wherein said control means comprises a selective control means for indicating specific applications software modules or groups of application software modules into which stack signature marking code segments are to be inserted.

Serial No. 10/047,011

Lorin Ullmann

Page 7 of 28

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Claim 16 (new):

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The method as set forth in Claim I further comprising encrypting at least a portion of said stack signature.

Claim 17 (new):

The method as set forth in Claim 2 further comprising encrypting said instance number in said stack signature.

Claim 18 (new):

The method as set forth in Claim 1 wherein said step of pushing stack signatures onto said processing stack comprises:

generating a pseudo-random identifier for each object instance dynamically created during runtime of a re-entrant executable code module; and

including said pseudo-random identifier in said stack signature pushed onto said processing stack.